Compartive Study of Volatile Components and Fatty Acids of Plants and in Vitro Cultures of Parsley (*Petroselinum crispum* (Mill) Nym ex Hill)

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Volatile compounds from plants, callus tissue cultures, and cell suspensions of parsley (*Petroselinum crispum*) were captured during the growth cycle using a dynamic headspace extraction and were identified by gas chromatography−mass spectrometry. Parsley plants were found to produce mainly monoterpenes, and the compound of major abundance was *p*-1,3,8-menthatriene, followed by *β*-phellandrene and apiole. Callus cultures and cell suspensions produced aldehydes (nonanal and decanal) that were also detected in parsley plant. The former also produced limonene, acetophenone, and benzotiazol; these were not observed in the plants. The production of volatiles in plants, callus tissue, and cell suspensions was found to be time-dependent. Free and bound fatty acids were also monitored by an in situ method. Palmitic (C16:0) and stearic (C18:0) acids were the most abundant fatty acids in all materials; however, higher levels were found in plants. On the other hand, the unsaturated C16:1 and C16:3 were not detected in the in vitro cultures.

Keywords: *Parsley; Petroselinum crispum; callus; cell suspensions; volatiles; dynamic headspace; fatty acids; in situ; GC−MS*

INTRODUCTION

Parsley (*Petroselinum crispum*) is a member of the Umbelliferae family that has been employed in the food, pharmaceutical, perfume, and cosmetic industries mainly because of its essential oil content (Simon and Quinn, 1988; Leung and Foster, 1996). Volatile compounds of parsley that have been reported include myrcene, myristicin, *β*-phellandrene, α-pinene, and *p*-1,3,8-menthatriene (Kasting et al.,1972). MacLeod et al. (1985) reported 45 volatile compounds from parsley leaves, and the most abundant components were myristicin (20.6%), apiole (18.3%), *β*-phellandrene (12.4%), and *p*-1,3,8-menthatriene (9.2%). According to Simon and Quinn (1988), myrcene was found to be the most abundant volatile component (16%) of the essential oil extracted from leaves of different parsley cultivars. Changes in volatiles during the phenological phases of parsley have been also described, and sniffing analysis of the volatile components revealed that the characteristic parsley aroma was mainly due to *p*-1,3,8-menthatriene, *p*-dimethylstyrene, and *β*-phellandrene (Porter, 1989). A more recent gas chromatography−olfactometry analysis of volatile compounds from leaves demonstrated that 14 different components contribute to parsley aroma; among them, myristicine, myrcene, linalol, and 2-methyl butanoate were the most important (Jung et al., 1992). A very recent study on the most potent odorants of parsley has shown that 17 compounds are involved in the unique aroma of the cultivar (Masanetz and Grosch, 1998). The authors reported that *p*-1,3,8-menthatriene, myrcene, 2-*sec*-butyl-3-methoxypyrazine, myristicin, linalool, 6-decenal, and 3-hexenal were the character impact flavor compounds of parsley.

In vitro cultures of parsley have been used as model systems to study the production of secondary metabolites (Kreuzaler and Hahlbrock, 1973; Hahlbrock and Wellmann,1970). The number of reports on volatile components produced by in vitro cultures of parsley are very scarse, and the described compounds are 3-*n*-butylphthalide, sedanolide, sedanenolide, (*Z*)-lingustilide, *n*-alkanes, phthalates, and elemicine; however, microorganisms as elicitors were used during this study (Reil and Berger, 1996).

Fatty acids of parsley have only been studied in seeds (Privett et al., 1963). The major fatty acid found in seeds was C18:1, with the unsaturation on position 6 (petroselinic acid). This fatty acid accounted for almost 75% of bound and free fatty acids in parsley seeds. Other fatty acids found in this system were oleic and petroselinic (82%), linoleic (13%), palmitic (5%), and palmitoleic (traces). In another investigation, Kleiman and Spencer (1982) reported the following percentages obtained on thin-layer chromatography: C16:0, 3.7; C18:0, 1.1; C18:1−6, 71.5; C18:1−9, 5.3; C18:2, 8.5; and C18:3, 9.3.

The present investigation was undertaken to study the differences or similarities in the production of volatile and fatty acid contents during the growth cycle of parsley plants and in vitro cultures.

MATERIALS AND METHODS

Plant Material. Parsley seeds (*Petroselinum crispum* var. *neapolitanum*) were sown at 0.5−1-cm planting depth in germination trays (5−6 seeds per cavity) containing water-wetted peatmoss and covered with plastic domes to maintain humidity (45−50%). Germination trays were maintained under greenhouse conditions for 35 days at temperatures ranging from 12−15 °C minimum to 30−35 °C maximum.

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Seedlings were transplanted into pots (1-L capacity; four seedlings per pot) containing sterilized organic soil and were kept under greenhouse conditions for 3 months. These plants were analyzed for volatile compounds and bound and free fatty acids at 5, 7, 9, 11, and 13 weeks.

In Vitro Seedlings. Seeds of parsley were washed extensively with tap water and detergent in order to eliminate dust and gross contaminants. After being rinsed, the seeds were treated with a 20% (v/v) commercial bleach solution containing 0.2% Tween 20 for 20 min under agitation, vacuum, and sterile conditions. The solution was discarded, and the seeds were rinsed with sterile distilled water until no foam was formed in the rinsing liquid by vigorous agitation. Groups of seeds (15−20) were placed in 120-mL glass bottles containing 20 mL of MS semisolid culture medium (Murashige and Skoog, 1962) without growth regulators, pH 5.8, gelled with 0.8% agar, and incubated under light conditions (16 h light/8 h dark, cold white fluorescent lamps, 50 mE m^{-2} s^{-1}) and at 25 °C. After 40 days of incubation, the seedlings were transferred to 500-mL glass bottles containing fresh MS medium for further development.

Callus Cultures. Stem and leaf tissues from in vitro plants (3 months old) were used as explants to establish callus cultures. Explants (1 cm in length) were inoculated in 120-mL glass bottles containing 20 mL of MS semisolid culture medium, supplemented with 1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8, gelled with 0.8% agar, and incubated under the same conditions described for in vitro germination of seeds. Callus tissue was formed after 4 weeks, and the calli were maintained by 30-day subcultures on the same culture medium and under the same incubation conditions.

Cell Suspension Cultures. Parsley cell cultures were initiated using 10 g of callus tissue (6 months old) inoculated in a 250-mL Erlenmeyer flask containing 100 mL of MS liquid medium, supplemented with 1 ppm of 2,4-D, pH 5.8, maintained under agitation (125 rpm) and light (16 h light/8 h dark, cool white fluorescent lamps, 30 mE m^{-2} s^{-1}) and at 25 °C. After 15 days of incubation, the culture medium was eliminated by decantation and was replaced by the same volume of fresh medium, and the cells were incubated under the same conditions. These cultures produced fine cell suspensions at the end of this 15-day period.

Dynamic Headspace Extraction. Volatiles from plants (5 g), callus (13.3−28.5 g), and cell suspensions (100 mL) were collected by dynamic headspace extraction. Caproic acid methyl ester was used as an internal standard (0.352 mg for plants and 0.088 mg for in vitro materials). The volatile compounds were trapped by a porous adsorption polymer (Tenax TA) by purging with N_2 at a rate of 5 mL/s. After 12 h, the column containing the Tenax (0.06 g) was removed from the headspace container, and the volatiles were desorbed with recently distilled and dried solvents; 6 mL of pentane/diethyl ether (1:1) was used. The extracts were concentrated to 25 mL in a Kuderna-Danish apparatus at 36 °C. The extracts of volatiles from plants, callus, and cell suspensions were performed three times each from different plants and tissue culture materials. Two-microliter aliquots of each extract were analyzed immediately in a tandem GC−MS system.

Free and Bound Fatty Acids by an in Situ Determination. Macerated plants, callus, and cell suspensions (50 mg) were dissolved in 500 μL of water, and heptadecanoic acid (0.05 mg) was added as an internal standard. Next, 100-μL aliquots were simultaneously hydrolyzed and derivatized to their methyl ester forms with 1 mL of $NaOH/CH_3OH$ 0.5 M for 10 min at 90 °C in a Pierce reacti-vial. Complete derivation was assured with 1 mL of BF_3 at 90 °C for 10 min. Methyl esters were recovered by extraction with hexane (2 × 1 mL) and dried over anhydrous Na_2SO_4. The extracts were evaporated to dryness and redissolved in 20 μL of isooctane. All determinations were done in triplicate.

Gas Chromatography−Mass Spectrometry Analysis. Volatiles and fatty acids were analyzed in a HP5890 Series II gas chromatograph equipped with a MS detector. A capillary column, HP-5MS, of 30-m × 0.25-mm-i.d. cross-linked 5% methyl phenyl silicone was used. The GC conditions for the volatile analysis were as follows: oven temperature, 40 °C for 3 min, to final temperature of 240 °C at a rate of 3 °C/min, and hold this temperature for 5 min; injector temperature, 180 °C; detector temperature, 250 °C; flow rate of the carrier gas (helium), 0.44 mL/min; pressure, 21 kPa; split, 30:1. Operating conditions for the fatty acids were as follows: carrier gas, helium, 0.56 mL/min; detector, 250 °C; injector, 200 °C; injection volume, 2 mL. The column was held for 3 min at 130 °C and programmed at 3 °C/min to a final temperature of 270 °C for 3 min. Mass spectra were acquired with the ionization energy at 70 eV and within a mass range of *m*/*z* 50−550. The identification of the volatile compounds present in the extracts was performed by matching each component with a mass spectra library and also with the mass spectra of some authentic compounds. Fatty acids were identified by means of their retention times, individual mass spectra, and comparison with the authentic standards.

RESULTS AND DISCUSSION

Volatiles in Plants. Figure 1 shows a typical total ion chromatogram of parsley plants at 7 weeks. The volatile compounds found in plants at five different growth stages are listed in Table 1. Seventeen volatiles were completely identified in all stages. It can be seen that *p*-1,3,8-menthatriene (0.62−398.8 ppm), β-phellandrene (44.9−255.1 ppm), and apiole (13.7−96.7 ppm) were the most abundant constituents in all stages, except for *p*-1,3,8-menthatriene, which was the lowest at 5 weeks. The generation of these compounds was time dependent. Kasting et al. (1972) and Freeman et al. (1975) have reported previously most of these compounds in parsley leaves, but pinenes, phellandrenes, and xylenes were among the most abundant in their reports.

The highest levels of myrcene and myristicin were observed at 7 weeks; this might be of relevance, since Jung et al. (1992) reported that these two compounds are the most potent volatiles responsible for the parsley aroma. The impact of these volatiles was established by an aroma extract dilution analysis (AEDA). Therefore, this stage (7 weeks) could represent the best period of time to harvest this species. Furthermore, at this same stage, *p*-1,3,8-menthatriene, β-phellandrene, and apiole presented the highest concentrations. These volatiles presented two maximum levels (7 and 11 weeks); these concentration changes might be conditional on the essential functions of the plant. For instance, apiole is known as the major seed volatile (Porter, 1989); this could explain the increment of this compound in the last stage.

Volatiles in Callus. Eleven compounds were detected and identified in the callus tissue cultures (data not shown). More volatiles were detected after 10 and 20 days. 2-Carene was the only monoterpene found in callus materials that was present in plants. On the other hand, aldehydes such as nonanal and decanal were also detected in the callus. However, syntheses de novo for many other volatiles, such as styrene, benzaldehyde, limonene, acetophenone, and benzothiazol, were observed. Most of these compounds have been found before in fresh parsley plants: *p*-xylene (Kasting et al., 1972; Freeman et al., 1975), styrene (Kasting et al., 1972), limonene (Garnero and Chrétien-Bessiére, 1968; Ikeda et al., 1962), and benzaldehyde (MacLeod et al., 1985). However, these volatiles were not present in any of the plant samples analyzed in this work. On the other hand, methylated styrene and acetophenone have been re-

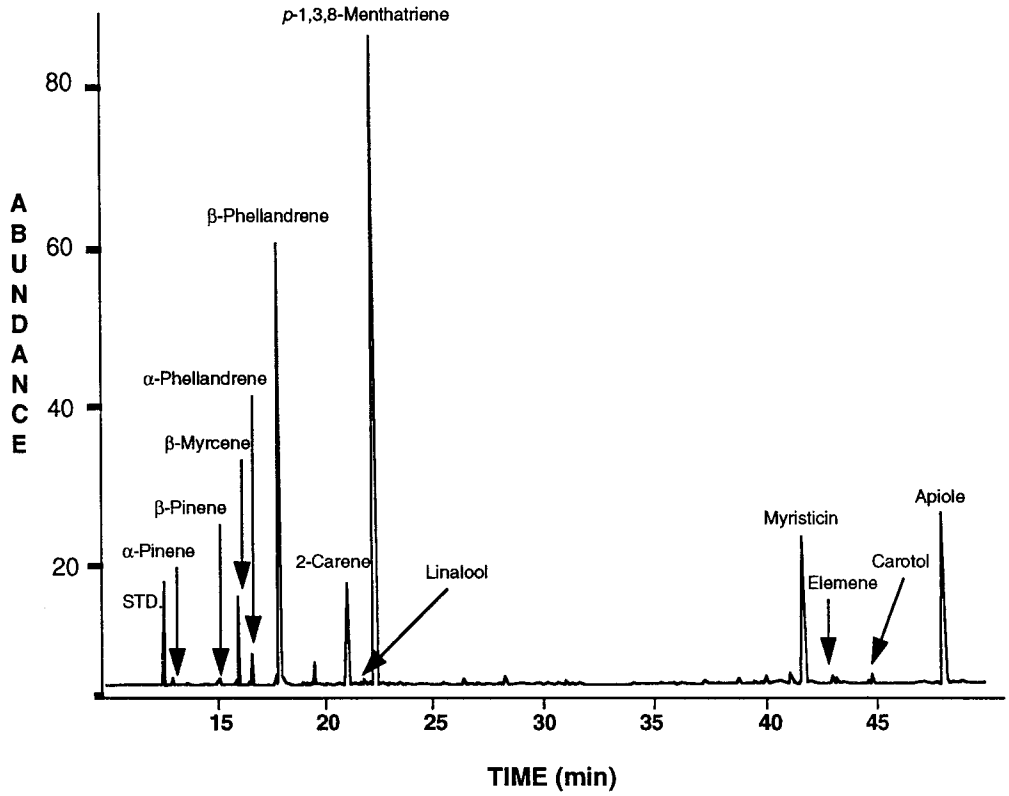

Figure 1. Volatile composition of parsley plants during growth cycle. Profile of the volatiles found at 7 weeks.

Table 1. Volatile Composition (ppm) of Parsley Plants during Growth Cycle[a]

		weeks				
volatiles	t_R[b]	5	7	9	11	13
α-pinene	12.95	0.88 (0.88)	6.30 (0.003)	27.62 (12.72)	9.40 (4.28)	17.76 (3.29)
β-pinene	15.10	2.44 (0.81)	5.20 (0.65)	18.39 (9.69)	12.42 (5.90)	4.82 (4.82)
β-myrcene	15.99	18.89 (2.02)	36.03 (28.96)	8.42 (5.32)	18.31 (3.11)	18.33 (8.01)
α-phellandrene	16.60	6.51 (1.88)	15.68 (8.55)	4.62 (1.06)	7.90 (3.91)	10.22 (5.76)
β-phellandrene	17.63	83.58 (31.39)	255.69. (131.70)	45.06 (6.92)	116.81 (65.39)	nd
2-carene	21.08	nd	56.94 (39.02)	14.35 (2.58)	nd	nd
linalool	21.86	0.73 (0.004)	5.17 (4.70)	nd	0.85 (0.38)	2.05 (2.05)
nonanal	21.99	0.68 (0.07)	nd	0.30 (0.30)	nd	nd
p-1,3,8-menthatriene	22.40	0.62 (0.62)	399.73 (315.48)	119.45 (38.18)	364.03 (187.56)	371.73 (202.02)
thymol	26.18	nd	nd	t	8.53 (7.92)	t
decanal	27.01	0.62 (0.036)	nd	0.76 (0.25)	nd	1.23 (0.67)
copaene	35.15	t	nd	0.32 (0.32)	1.16 (0.62)	3.09 (2.56)
caryophyllene	37.22	nd	nd	0.96 (0.96)	8.76 (5.26)	15.88 (14.17)
myristicin	41.74	17.16 (17.16)	84.16 (84.16)	nd	nd	nd
elemene	43.04	1.51 (1.52)	3.81 (3.81)	nd	nd	nd
carotol	44.82	0.49 (0.49)	4.06 (4.06)	nd	nd	nd
apiole	48.17	19.71 (4.25)	96.94 (91.89)	8.67 (3.38)	44.66 (25.00)	52.49 (41.77)

[a] t, traces; nd, not detected. Numbers in parentheses correspond to the standard errors. [b] Retention time.

ported also in parsley plants (Garnero and Chrétien-Bessiére, 1968; Vernon and Richard, 1983).

Volatiles in Cell Suspensions. Table 2 shows the volatiles found in the parsley cell suspensions. Fifteen volatile compounds were observed in these suspensions. Again, 2-carene, nonanal, and decanal were common to the plant material. The most productive stages were at 5 (0.797 ppm) and 10 days (0.463 ppm). However, the volatile found at the greatest level was decanal, with 0.680 ppm (5 days), followed by benzothiazol (10 days) and nonanal (10 days), with 0.171 and 0.083 ppm, respectively. Some volatiles not present in plant or callus samples were found in cell suspensions, such as terpenes (limonene, eucalyptol, and linalyl propanoate), aldehydes (hexanal, octanal, and undecanal), acetophenone, and benzothiazol. Berger (1995) and Reil and Berger (1996) reported the synthesis of butylphthalate, sedanenolide, sedanolide, elemicine, *n*-alkenes, and coumarins from cell suspensions of parsley; however, these investigators used microorganisms as elicitors, and this could be the explanation for the enormous differences among volatiles with the present work.

Free and Bound Fatty Acids in Plants. Tables 3−5 list all the fatty acids found in plants, callus tissue, and cell suspensions, respectively. In Table 3, it can be seen that C16 and C18 account for almost 100% of the bound and free fatty acids in plant materials. Among the major fatty acids, C18:3 and C18:2 were the most abundant in all stages. Fatty acid C16:3 was also present in all stages; the highest concentration was observed at 7 weeks. On the other hand, both C16:1 (position 7 and 9) were found only up to 9 weeks, with a clear optimum at 7 weeks. As was expected, petroselinic acid was not detected in any of the samples, since

Table 2. Volatile Compounds Found (ppm) in Parsley Cell Suspensions at Four Stages[a]

		days			
volatiles	t_R[b]	0	5	10	15
hexanal	7.12	nd	nd	nd	t
p-xylene	9.73	t	nd	nd	nd
octanal	16.63	nd	t	nd	t
limonene	17.86	nd	0.017 (0.017)	0.026 (0.026)	t
eucalyptol	18.05	nd	t	nd	nd
acetophenone	19.94	nd	0.030 (0.030)	0.067 (0.067)	t
2-carene	21.04	nd	0.035 (0.035)	t	t
nonanal	21.91	t	0.035 (0.035)	0.083 (0.083)	t
azulene	25.88	nd	t	nd	nd
naphthalene	25.90	nd	nd	t	nd
linalyl propanoate	26.44	nd	nd	nd	t
decanal	27.07	t	0.681 (0.60)	0.117 (0.117)	t
benzothiazol	28.04	t	t	0.171 (0.171)	t
undecanal	32.00	nd	t	nd	nd
1-hexacosanol	39.50	nd	t	nd	nd

[a] t, traces; nd, not determined. Numbers in parentheses correspond to the standard errors. [b] Retention time.

it is known to be present only in parsley seeds (Privett et al., 1963).

Fatty Acids in Callus. Ten fatty acids were found in callus tissue cultures of parsley (Table 4). All the fatty acids displayed an increasing behavior during the four analyzed stages. The highest levels for all fatty acids were observed at 30 days, where C16:0 (329 ppm), C18:2 (302 ppm), and C18:3 (106 ppm) were the most abundant. Unsaturated C16 was not detected in any of the sample. However, longer fatty acids such as C20:0 and C22:0 were found. There are no reports on the production of fatty acids of parsely plants or tissue cultures; however, very low levels of lipids have been reported in in vitro studies of *Artemisia absinthium*, *Brassica napus*, and *Hydnocarpus anthelminthica* (Radwan et al., 1975).

Fatty Acids in Cell Suspensions. As it was seen in callus tissue cultures, C16:0 and C18:2 were the most predominant fatty acids in cell suspensions. However, C18:3 was not detected in these in vitro systems, and fatty acids such as C14:0, C15:0, C20:0, and C22:0 were only found in traces. The most abundant fatty acid was C18:0 at day 0, with 309.77 ppm.

CONCLUSIONS

Many of the volatiles found in this work have been previously reported in studies on parsley leaves. However, in this work, the most abundant components were *p*-1,3,8-menthatriene and β-phellandrene (7 week). Besides, the most potent odorants responsible of the parsley aroma (myrcene and myristicin) were also found at this period. Callus tissue cultures and cell suspen-

Table 3. Fatty Acids (ppm) in Parsley Plants during Growth Cycle[a]

		weeks				
fatty acids	t_R[b]	5	7	9	11	13
C14:0	16.86	nd	t	13.12 (13.12)	t	t
C15:0	20.17	14.72 (14.72)	27.23 (27.23)	nd	15.40 (15.40)	nd
C16:3(7,10,13)	22.62	205.36 (23.03)	383.88 (48.79)	74.36 (74.36)	52.73 (52.73)	99.23 (10.05)
C16:1(7)	23.35	nd	47.55 (47.55)	22.10 (22.10)	nd	nd
C16:1(9)	23.38	48.98 (24.53)	71.95 (36.12)	nd	nd	nd
C16:0	23.52	647.45 (58.90)	800.21 (59.45)	600.86 (20.32)	557.12 (5.63)	247.97(25.67)
C18:2(9,12)	28.85	767.84 (40.38)	1011.09 (85.60)	680.52 (67.49)	593.64 (18.73)	294.19(30.91)
C18:3(9,12,15)	29.05	916.34 (71.46)	1346.65 (200.91)	847.24 (36.67)	654.73 (16.02)	405.59(38.88)
C18:0	29.85	109.65 (12.12)	98.01 (5.09)	96.05 (9.43)	66.01 (5.77)	nd

[a] t, traces; nd, not determined. Numbers in parentheses correspond to the standard errors. [b] Retention time.

Table 4. Fatty Acids (ppm) in Parsley Callus Tissue Cultures at Four Stages[a]

		days			
fatty acids	t_R[b]	0	10	20	30
C14:0	16.89	nd	t	21.49 (6.95)	24.19 (0.97)
C15:0	20.20	0.03 (0.03)	nd	nd	nd
C14:0 9-Me	20.33	nd	nd	10.93 (10.93)	nd
C16:0	23.53	0.22 (0.02)	0.31 (0.04)	262.50 (52.32)	329.56 (26.46)
C18:2(9,12)	28.85	0.07 (0.005)	0.23 (0.05)	191.22 (39.19)	301.72 (24.49)
C18:3(9,12 15)	29.03	nd	nd	42.03 (23.51)	106.28 (12.85)
C18:1(9)	29.05	nd	nd	30.96 (21.88)	nd
C18:0	29.85	0.08 (0.007)	0.07 (0.005)	52.50 (2.81)	76.21 (15.19)
C20:0	35.76	nd	nd	1.62 (1.62)	4.52 (4.51)
C22:0	41.24	nd	nd	nd	4.22 (4.22)

[a] t, traces; nd, not determined. Numbers in parentheses correspond to the standard errors. [b] Retention time.

Table 5. Fatty Acids (ppm) in Parsley Cell Suspensions at Four Stages[a]

		days			
fatty acids	t_R[b]	0	5	10	15
C14:0	16.88	t	t	t	t
C15:0	20.21	t	t	t	t
C16:0	23.50	183.18 (14.91)	283.61 (8.57)	281.81 (6.07)	163.06 (32.34)
C18:2(9,12)	28.84	95.47 (13.18)	232.13 (1.50)	209.89 (4.55)	200.24 (40.53)
C18:1(9)	29.03	nd	79.99 (3.38)	64.77 (2.43)	59.02 (19.67)
C18:0	29.85	309.76 (309.76)	15.90 (15.90)	10.78 (10.78)	t
C20:0	35.75	nd	t	t	nd
C22:0	41.25	nd	nd	t	t

[a] t, traces; nd, not determined. Numbers in parentheses correspond to the standard errors. [b] Retention time.

sions were able to generate some of the volatile compounds common to the plant material, such as aldehydes (nonanal and decanal) and some terpenes. Other potent compounds, such as benzaldehyde and benzothiazol, were also generated.

For the first time, free and bound fatty acids of parsley plants, callus tissue cultures, and cell suspension have been reported here. The concentrations of fatty acids found in the in vitro samples were not close to the levels in parsley plants; however, this information is highly valuable since unsaturated fatty acids, mainly C18:3, were synthesized.

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